

## Article

# Neem Leaf Extracts and Azadirachtin Trigger a Moderate Early Defense Response in Sunflowers Infected with Downy Mildew Caused by *Plasmopara halstedii* (Farl.) Berl. et de Toni

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**Abstract:** This study examined the effectiveness of neem leaf extract (NLE) and azadirachtin (AZA) against two isolates of *Plasmopara halstedii*, which causes downy mildew in sunflowers. We also explored their physiological and histopathological effects. The pre-inoculation treatments included 10% and 20% NLE and 0.01% and 0.1% AZA, compared to a mefenoxam-treated control and a non-treated control. All treatments significantly reduced the disease rate compared to the inoculated controls (which had a 73–76% disease rate). The 10% NLE treatment showed the strongest effect against isolate 1 (0% damping-off), while the 20% NLE treatment was most effective against isolate 2 (4% damping-off). Neem treatments also significantly improved plant height; for instance, 20% of NLE-treated plants inoculated with isolate 2 reached approximately 15 cm, compared to about 8 cm in the inoculated controls. Histological analyses indicated limited hyphal spread and low levels of cortical necrosis in neem-treated plants, particularly with 0.1% AZA treatment. This suggests a moderate initial defense response without extensive hypersensitive cell death. Neem treatments were comparable to mefenoxam treatments. These results highlight the potential of neem-derived products, particularly 10% NLE and 0.1% AZA, for the integrated management of sunflower downy mildew through both direct pathogen suppression and enhanced host resistance.

**Keywords:** sunflower downy mildew; neem-derived pesticides; hypersensitive reaction (HR); necrosis; hypersensitive cell death; host defense; tissue reaction; mefenoxam



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## 1. Introduction

The use of natural plant extracts to control various diseases (plant, animal, and human) has been reported [1–3]. However, in the context of plant protection, it is currently experiencing a resurgence [4]. Water or ethanol extracts from different parts (e.g., seeds, leaves, and kernels) of the neem tree (*Azadirachta indica* A. JUSS.) occupy a unique position among plant derivatives that have been utilized to control various pests [5]. Azadirachtin derivatives in neem trees offer novel tools for plant protection.

Neem is a versatile solution, mainly used against insects [6], but it has also proven effective against several plant pathogens [7]. Diverse neem extracts (and active ingredients) have been successfully used to combat leaf spot diseases in a wide range of crops, including rice [8], groundnut [9], sesame [10], chili [11], brinjal [12], eggplant [13], and barley [14]. The various active ingredients in neem have effectively protected tomatoes from Fusarium wilt [15–17] and bacterial wilt [18]. It has also been effective against powdery mildew in peas [19] and provides protection against downy mildew in grapes [20] and sunflowers [21].

Downy mildew has a global impact and causes significant damage to crops [22]. *Plasmopara halstedii* (Farl.) Berl. et de Toni, the causal agent of sunflower downy mildew, is particularly dangerous for this crop [23]. It can precipitate dwarfing, leaf chlorosis, and, in severe cases, damping off (plant death). Chemical control is ineffective once systemic symptoms emerge during early infection [23,24]. Due to its high variability, the pathogen's capacity to expeditiously generate different variants (pathotypes) presents a complex epidemiological challenge [25–27]. These variants can easily overcome the dominant resistance genes in sunflower hybrids and develop resistance to fungicides used as seed treatments [25,27]. This highlights the need for collaboration among researchers, farmers, and policymakers to identify effective control options against sunflower downy mildew.

Disease resistance is a crucial aspect of a plant's defense system, and the hypersensitive reaction (HR) plays an essential role in this process [28]. HR results from numerous biochemical and cellular processes, including the rapid increase in reactive oxygen species, nitrogen monoxide, and genes related to pathogenesis and defense mechanisms. When a plant identifies an invading pathogen, this recognition typically precipitates rapid, localized cell death (cell necrosis) in the infected area, which prevents pathogen dissemination. As a result, HR is vital for enhancing the plant's immune response. However, hypersensitive reactions should be conceptualized as a dynamic interplay between resistance and cell death (necrosis), where cell death is not necessarily required for effective resistance [29,30]. Neem extracts and products effectively combat phytopathogens through various mechanisms. They can directly inhibit pathogen growth and prevent its establishment and propagation in the host plant [4]. Additionally, neem may be critical in successfully eliciting systemic acquired resistance (SAR), thereby enhancing the overall resilience of the plant [7].

Botanical pesticides, such as neem derivatives, are novel solutions for integrated pest management. However, before they can be widely adopted, a precise understanding of their mechanism of action is essential [31], with histopathological studies being an indispensable element. Hypersensitive reactions and cell necrosis due to infection are relatively well-detectable tissue manifestations of plant defense reactions. Some authors have investigated these responses in susceptible and genetically resistant sunflowers infected with downy mildew [32–36] and in sunflowers treated with inducers [37,38]. Although the efficacy of neem pesticides against sunflower downy mildew has been investigated [21,39], no detailed data on the processes that occur in plant tissues are available.

Therefore, our research aimed to:

1. Evaluate the effectiveness of neem leaf extract and its active compound, azadirachtin, in laboratory experiments against two distinct isolates of *P. halstedii* in sunflowers.
2. Assess the physiological responses of inoculated sunflower plants treated with neem-based products, focusing on alleviating disease symptoms and signs, such as sporulation on the cotyledons, damping-off, stunted growth, and leaf chlorosis.
3. Explore the preliminary histopathological changes in sunflower tissues inoculated with downy mildew after treatment with neem derivative. This will provide insights into host-pathogen interactions and the potential mechanisms of disease suppression and host response.

Neem is well-known for its broad-spectrum pesticidal properties; however, its effectiveness in controlling oomycete pathogens in oilseed crops, like sunflowers, has not been thoroughly studied. Additionally, our histological investigations are the first to show that neem-treated sunflowers mount an effective response to infection by two aggressive *P. halstedii* isolates that differ in virulence.

## 2. Materials and Methods

### 2.1. Origin and Propagation of *Plasmopara halstedii* Inoculum

The selected isolates of *P. halstedii*, stored at  $-70^{\circ}\text{C}$ , were part of the Department of Integrated Plant Protection of the Hungarian University of Agriculture and Life Sciences collection. Isolates were chosen for the studies based on their virulence and aggressiveness. Isolate 1 (from Mád, Hungary) was classified as pathotype 700 (less virulent), and isolate 2 (from Rákóczi falva, Hungary) was classified as pathotype 704 (highly virulent), based on previous studies [27]. Furthermore, suitable fitness (e.g., good reproductive potential and short incubation period) is an essential parameter in the selection of isolates. According to previous studies, the incubation period of the isolates, defined as the number of days from infection to sporulation, was consistently 8 days. In both instances, over half of the infected plants died. The short incubation period and high damping-off rate indicate that the two isolates used in this experiment were highly aggressive.

Inoculum preparation, inoculation, and cultivation of inoculated plants were performed according to Trojanová et al. [40]. Accordingly, the sporangia were washed off the frozen and infected leaves in bidistilled water to increase the concentration of the inoculum. The inoculum concentration was adjusted to 50,000 sporangia per mL using a Burkholder chamber. Sunflower seeds were surface sterilized in a 1.5% NaOCl solution for 5 min and germinated at  $21^{\circ}\text{C}$  for three days between filter papers. After washing the seedlings in running tap water, inoculation was performed using the whole seedling immersion (WSI) method [41] by suspending the seedlings in the inoculum at  $16^{\circ}\text{C}$  overnight in the dark. Then, 1 mL of the inoculum was used per seedling in a Petri dish.

Inoculated sunflower seedlings were planted in horticultural perlite and placed in a growth chamber at  $22^{\circ}\text{C}$ , with 12 h of light per day and a light intensity of  $100\ \mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  (Figure 1a). Once the first pair of true leaves appeared (9 days post-inoculation, 9 dpi), the sunflowers were kept in a dark environment at  $19^{\circ}\text{C}$  with 100% relative humidity for 24 h to promote sporulation. The collected sporangia were used as inocula for the experiments.

### 2.2. Preparation of Neem Leaf Extract (NLE) and Azadirachtin (AZA)

Neem leaf extract was prepared following the method described by Doshi et al. [42] with minor modifications. Briefly, air-dried neem leaves procured from Mumbai, India, were transported to the Department of Integrated Plant Protection in Gödöllő, Hungary. The neem leaves were removed from the package, kept at room temperature, and ground into a powder using an electric blender. Ten and 20% (*w/v*) neem leaf powder solutions were prepared by soaking 10 and 20 g neem leaf powder in 100 mL distilled water overnight in the dark. Post-storage, the mixture was filtered and centrifuged at 5000 rpm for 5 min to obtain a clear neem leaf extract solution. Furthermore, 0.01% and 0.1% working solutions were prepared using NeemAzal T/S (Trifolio GmbH, Lahnau, Germany), which contained 1% azadirachtin. NeemAzal is a registered commercial plant protection product in the European Union. Working solutions were prepared by dissolving 1 mL and 10 mL of NeemAzal T/S in 100 mL of distilled water, according to Doshi et al. [21].

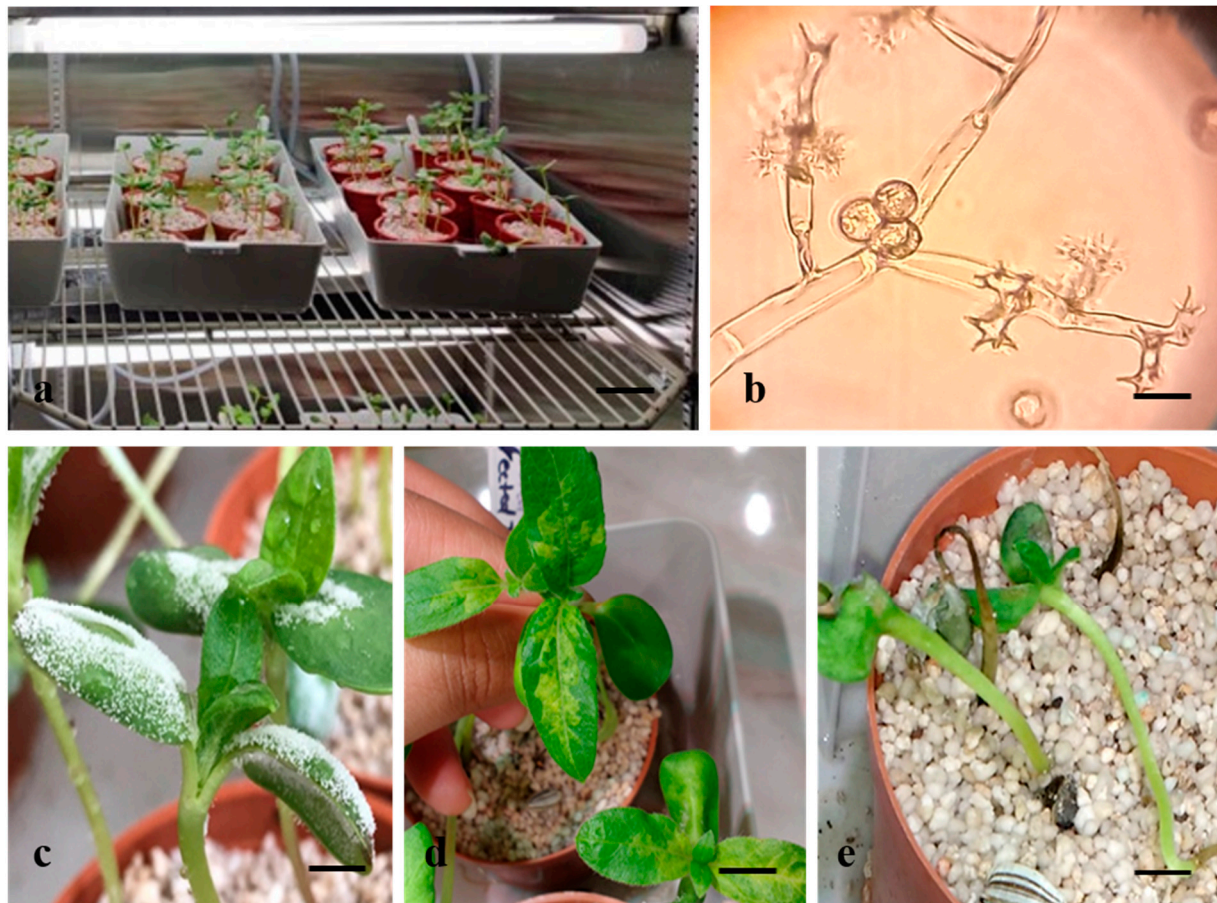


**Figure 1.** (a) Experimental design showing plants in the growth chamber, (b) micrographs of sporangia and sporangiophore, and (c–e) signs and symptoms of *Plasmopara halstedii* on non-treated and inoculated plants: (c) sporulation on cotyledons (9 dpi); (d) chlorosis on true leaves (21 dpi); (e) damping-off (post-emergence, 21 dpi) (Photos: (a,c–e): N. Nisha, (b): R. Bán). Scale bars: (a) = 4 cm, (b) = 25  $\mu$ m, (c) = 0.25 cm, (d) = 0.6 cm, and (e) = 1 cm.

### 2.3. Preparation of Mefenoxam

Mefenoxam (350 g a. i./L in Apron XL 350 FS (Syngenta, Budapest, Hungary), a fungicidal active ingredient previously registered against sunflower downy mildew, served as a positive control in the experiment, as in previous tests [21]. To prepare the mefenoxam solution, Apron XL 350 FS (Syngenta, Budapest, Hungary) was used at the EU-registered rate of 3 mL/Kg of seeds. The seeds were evenly coated with the solution and allowed to dry at room temperature for three days.

### 2.4. Set-Up of Experiment

In the experiment, a sunflower genotype (cv. Iregi szürke csíkos) susceptible to all pathotypes of *P. halstedii* was used. Seeds were sterilized and inoculated following the detailed procedure outlined in the “Origin and propagation of *Plasmopara halstedii* inoculum” chapter in the Methods and Materials section (see above). Neem-derived pesticide treatments were performed before inoculation by immersing the seedlings for two hours in the prepared solutions of the test agents. The control seeds were suspended in distilled water simultaneously with neem-derived pesticide treatment and in bidistilled water instead of being inoculated. The following 12 seed treatments and codes were applied to both *P. halstedii* isolates (isolates 1 and 2):

- 0 control: non-treated and non-inoculated with *P. halstedii*
- PH: non-treated and inoculated with *P. halstedii*

- MEF: treated with mefenoxam (3 mL/Kg) and non-inoculated with *P. halstedii*
- MEF + PH: treated with mefenoxam (3 mL/Kg) and inoculated with *P. halstedii*
- NLE 10%: treated with 10% neem leaf extract and non-inoculated with *P. halstedii*
- NLE 10% + PH: treated with 10% neem leaf extract and inoculated with *P. halstedii*
- NLE 20%: treated with 20% neem leaf extract and non-inoculated with *P. halstedii*
- NLE 20% + PH: treated with 20% neem leaf extract and inoculated with *P. halstedii*
- AZA 0.01%: treated with 0.01% azadirachtin (in 1% NeemAzal T/S) and non-inoculated with *P. halstedii*
- AZA 0.01% + PH: treated with 0.01% azadirachtin (in 1% NeemAzal T/S) and inoculated with *P. halstedii*
- AZA 0.1%: treated with 0.1% azadirachtin (in 10% NeemAzal T/S) and non-inoculated with *P. halstedii*
- AZA 0.1% + PH: treated with 0.1% azadirachtin (in 10% NeemAzal T/S) and inoculated with *P. halstedii*

After inoculation with isolates 1 and 2, treated and non-treated (and non-inoculated) seeds were planted in pots (diameter = 8 cm, five seeds per pot, 10 pots per treatment) filled with clean and moistened horticultural perlite (Figure 1a). Fifty seeds were used for each treatment. The plants were then placed in a growth chamber for 21 days under the circumstances described in the “Origin and propagation of *Plasmopara halstedii* inoculum” section.

## 2.5. Disease Assessment

The ratio of diseased to healthy plants was used to calculate the disease rate (%). Disease symptoms and signs were assessed twice: first, after the promotion of sporulation (9 dpi) based on the sporulated and pre-emergence damped-off (died) sunflowers, and second, according to the occurrence of systemic symptoms (leaf chlorosis and severe dwarfing) and damping-off (post-emergence) at 21 days post-inoculation (21 dpi). We also measured the height of the plants on both occasions, as dwarfing is a symptom of sunflower downy mildew.

## 2.6. Histopathological Studies

Histological examinations were performed on cross-sections of sunflower hypocotyls using a light microscope (Olympus, Tokyo, Japan). At 21 dpi, five sunflower hypocotyls from each treatment were selected and fixed in FAA solution (formalin: acetic acid: ethanol, 10:5:50 by volume). Thin cross-sections (20 pieces/hypocotyl) were cut with a razor blade from both the upper and lower parts of the hypocotyl and examined for pathogen structures (hyphae and haustoria) and tissue responses (cell necrosis, defined as hypersensitive cell death). As haustoria are only visible at higher magnification and forms rapidly after the appearance of hyphae, we only recorded the presence of hyphae under the microscope for rapid detection. The presence of dead cells determined necrosis. Following the method described by Bán et al. [37], a scale ranging from 0 to 4 was used to measure the extent of pathogen structures and host tissue responses.

## 2.7. Statistical Analysis

Two independent biological replicates were performed. Differences in disease rates, plant height, and host tissue responses (cell necrosis) were assessed using one-way analysis of variance (ANOVA) followed by the Tukey HSD (honestly significant difference) multiple comparison post hoc test. Levene’s test was applied to determine whether the variances were equal. The Spearman correlation coefficient was used to examine the correlations

between ordinal (microscopic) variables [43]. Minitab 18 statistical software was used for the statistical analysis.

### 3. Results

#### 3.1. The Efficacy of Neem-Derived Pesticides on Symptoms and Signs Caused by *Plasmopara halstedii*

After 9 days post-inoculation, the pathogen's asexual propagules (sporangiophores and sporangia, Figure 1b) were visibly present on both sides of the cotyledons as a white coating (Figure 1c) of the inoculated (treated and non-treated) plants following the induction of sporulation. At 21 days post-inoculation, chlorotic lesions were visible along the leaf veins, especially on the true leaves of the non-treated plants (Figure 1d). Damping-off occurred to a greater or lesser extent in the inoculated plants (Figure 1e).

On both recording dates for both isolates, there were significant differences in disease rates between the treated and non-treated plants, with the latter showing higher rates (Figure 2). However, inoculated plants treated with various concentrations of neem-derived pesticides or mefenoxam did not show significant differences from each other in disease rates at any time. Infection levels did not increase significantly by the second sampling date for any plant (treated and non-treated); NLE 10%-treated sunflowers inoculated with isolate 1 even exhibited a significant decrease in disease rate.

A similar trend to the level of infection was recorded in the proportion of damped-off (died) plants (Table 1). This indicates that significantly more untreated plants died than treated ones. At the same time, there was no significant difference in damping-off among sunflowers treated with different neem-derived pesticide concentrations. Furthermore, the damping-off rate of neem-treated plants was not significantly different from that of mefenoxam-treated plants. However, it is remarkable that a significant proportion of diseased plants, more than 50%, died in most cases. The exception was the plants treated with NLE 10% and NLE 20%, inoculated with isolates 1 and 2, respectively.

**Table 1.** Effect of two concentrations of neem leaf extract (NLE) and azadirachtin (AZA, in NeemAzal T/S), respectively, on pre- and post-emergence damping-off (%) of treated and non-treated sunflowers inoculated with *Plasmopara halstedii* at 21 days post-inoculation.

| Treatment      | Damping-Off<br>(% of Total Plants) |                          | Damping-Off<br>(% of Diseased Plants) |           |
|----------------|------------------------------------|--------------------------|---------------------------------------|-----------|
|                | Isolate 1                          | Isolate 2                | Isolate 1                             | Isolate 2 |
| PH             | 59 ± 9.5 <sup>a</sup>              | 64 ± 11.3 <sup>a</sup>   | >50                                   | >50       |
| MEF + PH       | 26 ± 4.8 <sup>b</sup>              | 12 ± 8.4 <sup>b</sup>    | >50                                   | >50       |
| NLE 10% + PH   | 0 <sup>b</sup>                     | 14 ± 8.2 <sup>b</sup>    | 0                                     | >50       |
| NLE 20% + PH   | 28 ± 9.6 <sup>b</sup>              | 4 ± 4.2 <sup>b</sup>     | >50                                   | 50>       |
| AZA 0.01% + PH | 16 ± 7.8 <sup>b</sup>              | 22.5 ± 12.4 <sup>b</sup> | >50                                   | >50       |
| AZA 0.1% + PH  | 22 ± 7.3 <sup>b</sup>              | 15 ± 8.8 <sup>b</sup>    | >50                                   | >50       |

Legend: see Figure 2. The individual standard deviations are shown after the mean values. According to Tukey's test, different letters (a, b) indicate a significant difference at 95% confidence.

The heights of sunflowers treated with different neem pesticides, with or without *P. halstedii* isolate 1 inoculation was not statistically different from the zero control (non-treated non-inoculated) and mefenoxam controls (MEF and MEF + PH) (Figure 3a). However, sunflowers treated with either neem or mefenoxam grew significantly taller than the non-treated and inoculated plants (PH). A similar pattern was observed for plants inoculated with isolate 2, except that no significant differences were found between the height of AZA

0.01% treated, inoculated, and non-treated inoculated plants (Figure 3b). Furthermore, inoculated plants (with isolate 2) treated with NLE 20% grew significantly taller than those that received the other treatments.

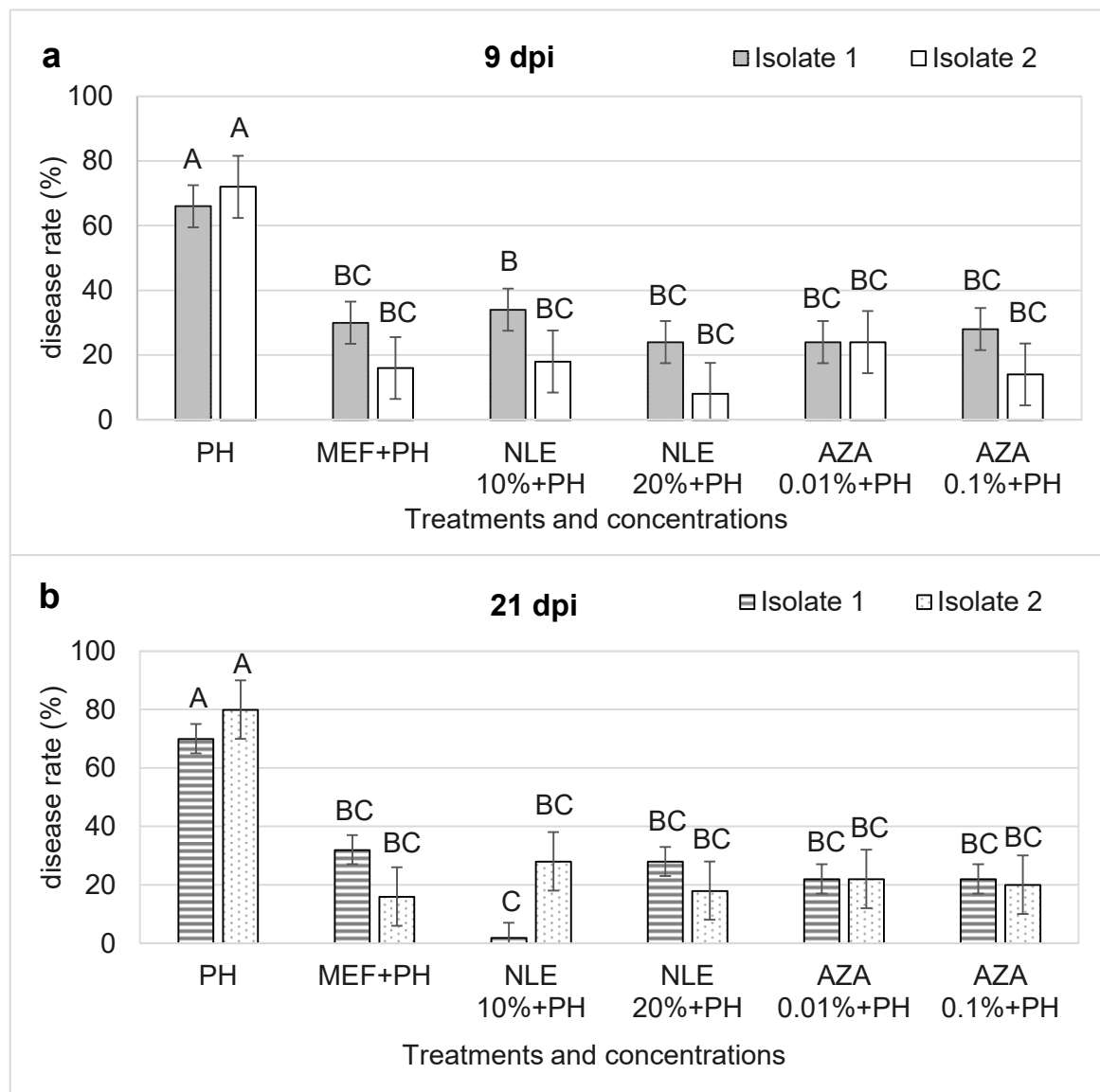


**Figure 2.** Effect of two concentrations of neem leaf extract (NLE) and azadirachtin (AZA, in NeemAzal T/S) on the disease rates (%) of sunflowers (a) 9 days and (b) 21 days after inoculation with *Plasmopara halstedii*. Legend: dpi: days post-inoculation; isolate 1: Mád isolate; isolate 2: Rákóczifalva isolate; PH: non-treated and inoculated with *P. halstedii*; MEF + PH; treated with mefenoxam (3 mL/Kg) and inoculated with *P. halstedii*; NLE 10% + PH: treated with 10% neem leaf extract and inoculated with *P. halstedii*; NLE 20% + PH: treated with 20% neem leaf extract and inoculated with *P. halstedii*; AZA 0.01% + PH: treated with 0.01% azadirachtin (in 1% NeemAzal T/S) and inoculated with *P. halstedii*; AZA 0.1% + PH: treated with 0.1% azadirachtin (in 10% NeemAzal T/S) and inoculated with *P. halstedii*. Mefenoxam was used as the positive control. According to Tukey's test, different letters indicate a significant difference at 95% confidence. The standard deviations are shown on the bars (n = 50 plants/treatment).

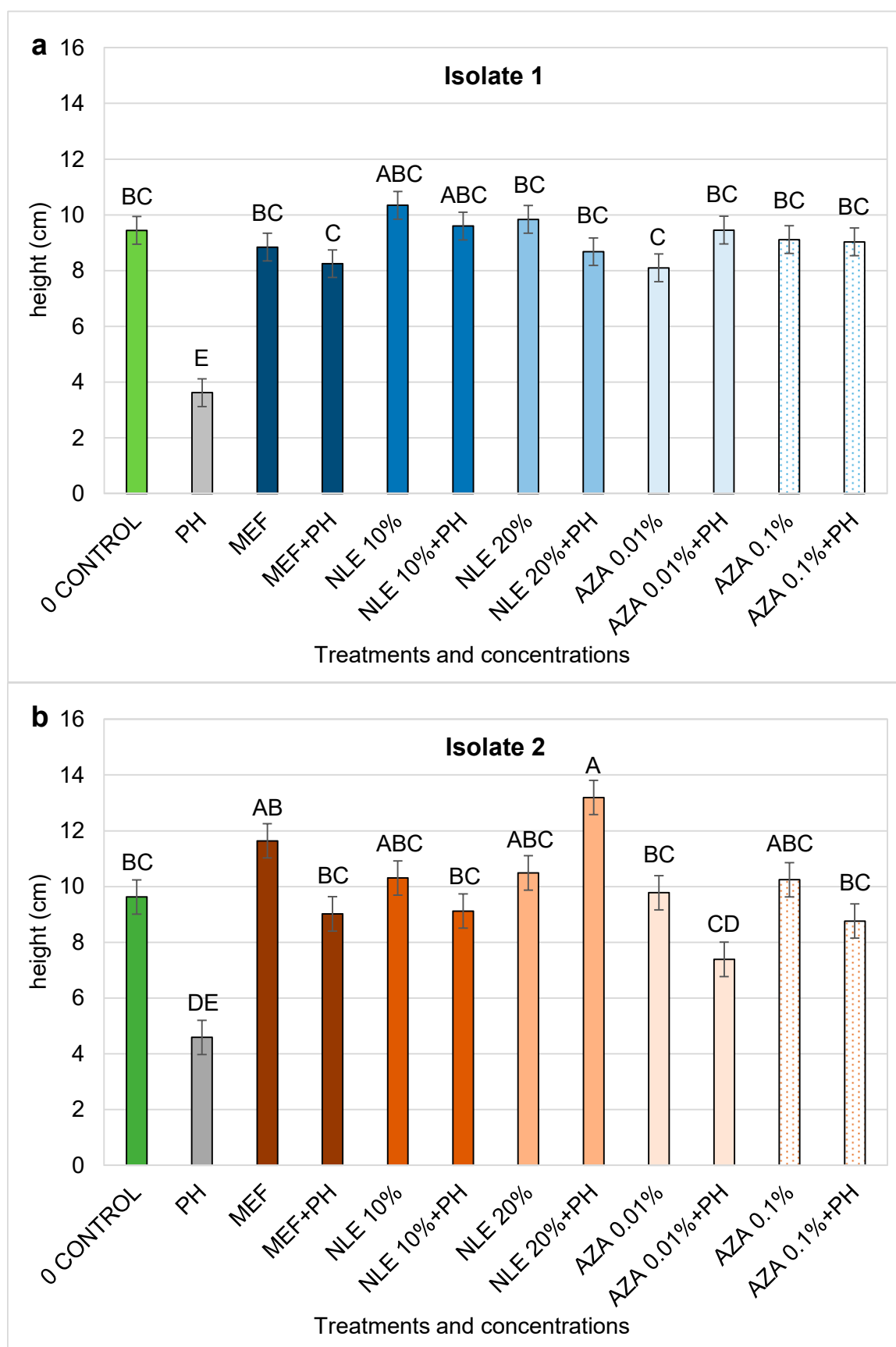


**Figure 3.** Effect of neem leaf extract (NLE) and azadirachtin (in NeemAzal T/S) on the plant height of sunflowers 21 days after inoculation with *Plasmopara halstedii* (a) isolate 1 and (b) isolate 2. Legend: isolate 1: Mád isolate; isolate 2: Rákóczifalva isolate; 0 control: non-treated and non-inoculated; PH: non-treated and inoculated with *P. halstedii*; MEF: treated with mefenoxam (3 mL/Kg) and non-inoculated

with *P. halstedii*; MEF + PH: treated with mefenoxam (3 mL/Kg) and inoculated with *P. halstedii*; NLE 10%: treated with 10% neem leaf extract and non-inoculated with *P. halstedii*; NLE 10% + PH: treated with 10% neem leaf extract and inoculated with *P. halstedii*; NLE 20%: treated with 20% neem leaf extract and non-inoculated with *P. halstedii*; NLE 20% + PH: treated with 20% neem leaf extract and inoculated with *P. halstedii*; AZA 0.01%: treated with 0.01% azadirachtin (in 1% NeemAzal T/S) and non-inoculated with *P. halstedii*; AZA 0.01% + PH: treated with 0.01% azadirachtin (in 1% NeemAzal T/S) and inoculated with *P. halstedii*; AZA 0.1%: treated with 0.1% azadirachtin (in 10% NeemAzal T/S) and non-inoculated with *P. halstedii*; AZA 0.1% + PH: treated with 0.1% azadirachtin (in 10% NeemAzal T/S) and inoculated with *P. halstedii*. Mefenoxam was used as the positive control. According to Tukey's test, different letters indicate a significant difference at 95% confidence. The pooled standard deviations are shown on the bars.

### 3.2. Histopathological Analysis of the Neem-Induced Resistance Through Some Host Responses

In most inoculated plants, pathogen hyphae were found in the intercellular spaces (Figure 4a), and haustoria were observed inside the cells (Figure 4b). Cell necrosis due to infection was also observed (Figure 4c) in inoculated plants. Around the necrotic areas, the cells elongated and divided.

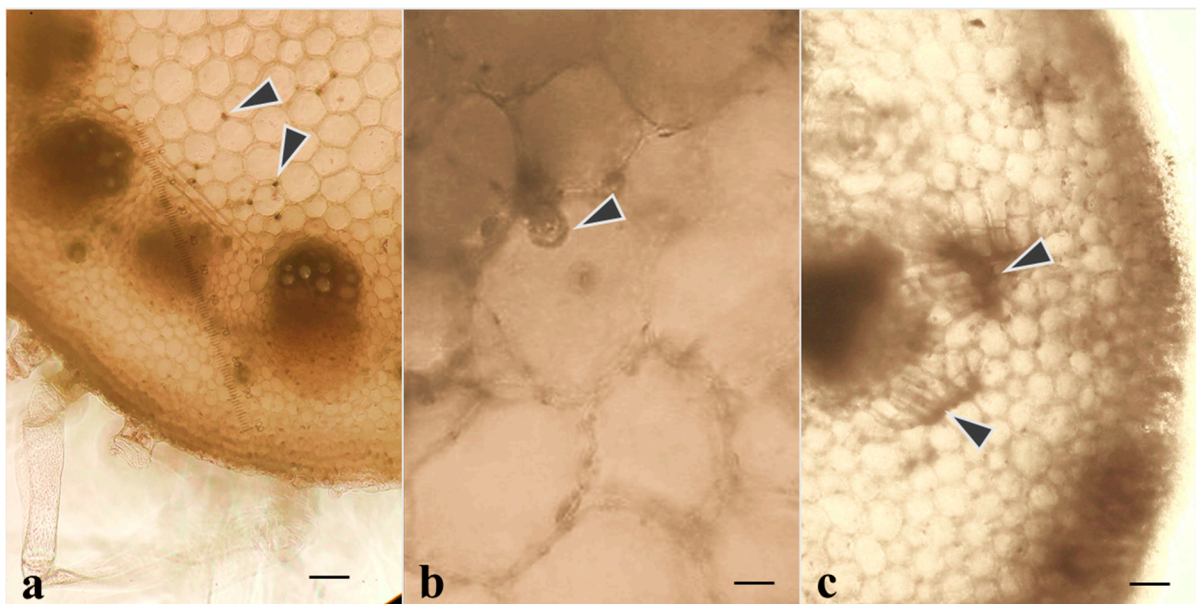


**Figure 4.** Light micrographs of (a) inter-cellular hyphae and (b) haustoria in a non-treated and inoculated (isolate 2) sunflower hypocotyl; (c) host cell necrosis in AZA 0.01% treated and inoculated (isolate 2) plant at 21 dpi. Arrows indicate pathogen elements (hyphae and haustoria) and host tissue reactions (necrosis). Scale bars: (a) 50  $\mu$ m; (b) 10  $\mu$ m; (c) 50  $\mu$ m (Photos: (a) N. Nisha; (b,c) R. Bán).

The appearance of the pathogen structures and tissue reactions varied significantly throughout the experiment. For both isolates of *P. halstedii*, the pathogen hyphae were predominantly observed in the cortical tissue and less frequently in the pith parenchyma, with necrosis exclusively found in the cortical part of the sunflower hypocotyls (Figure 5). In particular, for isolate 1, there was a significant increase in hyphal development in the cortical tissue of plants treated with mefenoxam (MEF + PH) and 0.1% azadirachtin (AZA 0.1% + PH) compared to the control plants (PH) (Figure 5a). On the contrary, the pathogen spread in the cortical tissue in the rest of the treated and inoculated plants was similar to that of the non-treated inoculated control (PH).

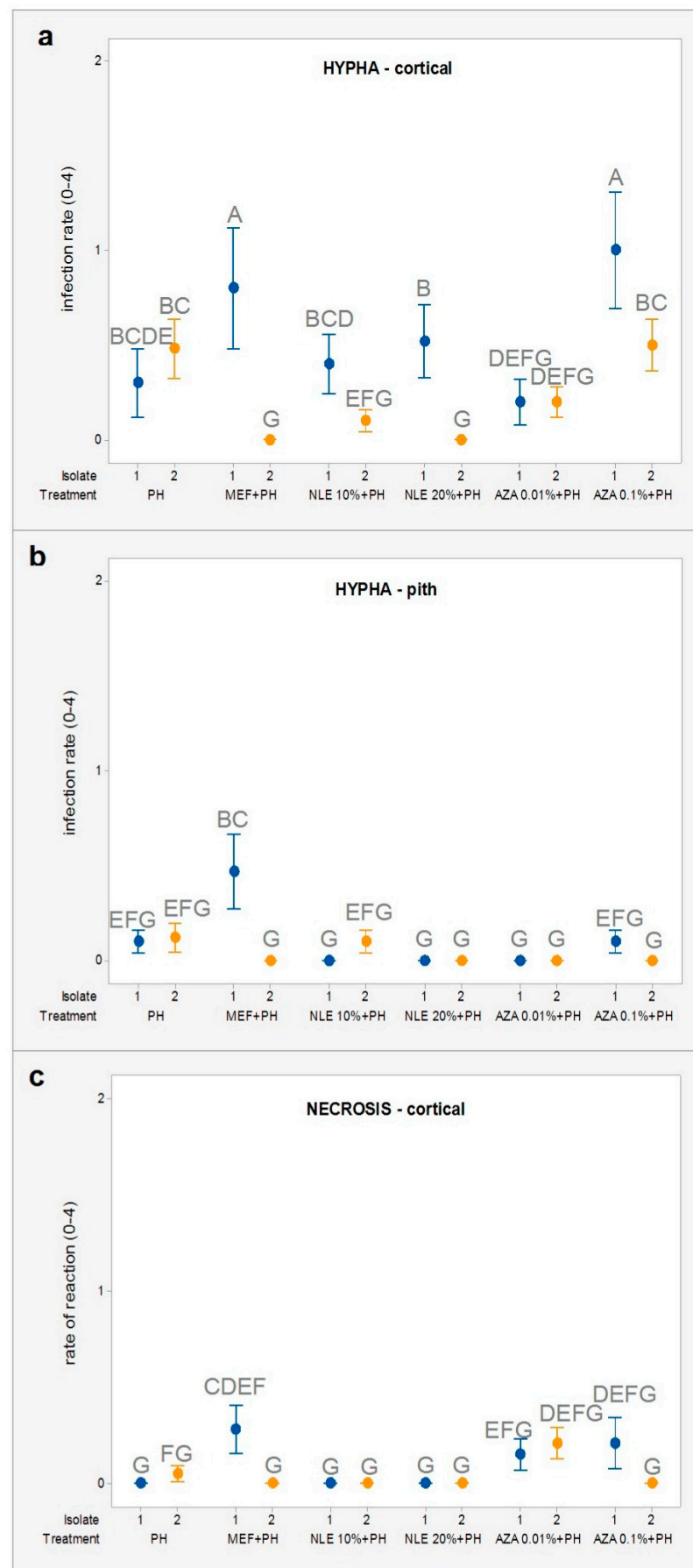


**Figure 5.** Effect of neem leaf extract (NLE) and azadirachtin (AZA, in NeemAzal T/S) on the occurrence of (a,b) pathogen hyphae and (c) host reaction, such as necrosis, in the cortical and pith

parenchyma of sunflower plants inoculated with *Plasmopara halstedii* 21 days post-inoculation. Legend: cortical: cortical parenchyma; pith: pith parenchyma; isolate 1: Mád isolate; isolate 2: Rákóczi falva isolate; PH: non-treated and inoculated with *P. halstedii*; MEF + PH: treated with mefenoxam (3 mL/Kg) and inoculated with *P. halstedii*; NLE 10% + PH: treated with 10% neem leaf extract and inoculated with *P. halstedii*; NLE 20% + PH: treated with 20% neem leaf extract and inoculated with *P. halstedii*; AZA 0.01% + PH: treated with 0.01% azadirachtin (in 1% NeemAzal T/S) and inoculated with *P. halstedii*; AZA 0.1% + PH: treated with 0.1% azadirachtin (in 10% NeemAzal T/S) and inoculated with *P. halstedii*. Mefenoxam was used as the positive control. The infection rate and host reaction rate were measured on a 0–4 scale. Vertical lines represent the 95% confidence intervals of the mean values. According to Tukey’s test, different letters indicate a significant difference at 95% confidence.

Interestingly, isolate 1 exhibited a higher abundance of hyphae than isolate 2 in the cortical tissue of treated sunflowers, except in the AZA 0.01% treated and control plants (PH). For isolate 2, hyphae spread in the cortical tissue was significantly higher in the non-treated inoculated plants (PH) and AZA 0.1% treated inoculated plants than in the other treatments (Figure 5a).

The distribution of pathogen hyphae in the pith parenchyma was similar in plants infected with different isolates and treated with various chemicals (Figure 5b). The only exceptions to this were plants treated with mefenoxam (MEF + PH) and inoculated with *P. halstedii* isolate 1, in which the spread of hyphae in the pith parenchyma was significantly greater than in the other plants. Several treatments applied before inoculation (MEF, AZA 0.1%, AZA 0.01%) resulted in necrosis in the plant cortical parenchyma, which was most pronounced in mefenoxam-treated plants (Figure 5c). However, some treatments (e.g., NLE 10% and 20%) did not result in necrosis in hypocotyls after infection with any *P. halstedii* isolates.

To better understand the microscopic results, we used Spearman’s correlation to explore potential connections between different elements, such as pathogen structures and plant tissue reactions (Table 2). A strong or moderate positive correlation was found in the appearance of pathogenic hypha between the cortical and pith parenchyma, independent of the *P. halstedii* isolate used, in non-treated inoculated plants (PH). This consistent relationship was also observed in plants treated previously with mefenoxam (MEF + PH) and inoculated with isolate 1 and in plants treated with 10% neem leaf extract (NLE 10% + PH) and inoculated with isolate 2. However, the correlation between cortical and pith hyphae was relatively weak in plants treated with AZA 0.1% and inoculated (isolate 1). In terms of tissue reactions, we found only weak but statistically significant negative correlations between the presence of hyphae in the parenchyma tissues and the appearance of necrosis in some of the treated plants (MEF + PH, AZA 0.01% + PH, and AZA 0.1% + PH). However, it should be stressed that there were marked differences between plants inoculated with the different isolates with respect to histopathological data.

**Table 2.** Spearman correlation among the examined microscopic variables (hyphae and necrosis).

| Variables/Treatments<br>(n = 100) | Isolate 1    |          | Isolate 2    |          |
|-----------------------------------|--------------|----------|--------------|----------|
|                                   | H-pith       | Nec-cort | H-pith       | Nec-cort |
| PH/H-cort                         | <b>1.000</b> | 0        | <b>0.543</b> | 0.183    |
| PH/H-pith                         | -            | 0        | -            | −0.076   |
| MEF + PH/H-cort                   | <b>0.994</b> | −0.249   | 0            | 0        |
| MEF + PH/H-pith                   | -            | −0.247   | -            | 0        |

Table 2. Cont.

| Variables/Treatments<br>(n = 100) | Isolate 1    |               | Isolate 2    |               |
|-----------------------------------|--------------|---------------|--------------|---------------|
|                                   | H-pith       | Nec-cort      | H-pith       | Nec-cort      |
| NLE 10% + PH/H-cort               | 0            | 0             | <b>1.000</b> | 0             |
| NLE 10% + PH/H-pith               | -            | 0             | -            | 0             |
| NLE 20% + PH/H-cort               | 0            | 0             | 0            | 0             |
| NLE 20% + PH/H-pith               | -            | 0             | -            | 0             |
| AZA 0.01% + PH/H-cort             | 0            | −0.032        | 0            | <b>−0.196</b> |
| AZA 0.01% + PH/H-pith             | -            | 0             | -            | 0             |
| AZA 0.1% + PH/H-cort              | <b>0.264</b> | <b>−0.263</b> | 0            | 0             |
| AZA 0.1% + PH/H-pith              | -            | −0.111        | -            | 0             |

Legend: n: number of examined cross-sections per treatment; H-cort: hypha in the cortical parenchyma; H-pith: hypha in the pith parenchyma; Nec-cort: necrosis in the cortical parenchyma; PH: non-treated and inoculated with *Plasmopara halstedii*; MEF + PH: treated with mefenoxam (3 mL/Kg) and inoculated with *P. halstedii*; NLE 10% + PH: treated with 10% neem leaf extract and inoculated with *P. halstedii*; NLE 20% + PH: treated with 20% neem leaf extract and inoculated with *P. halstedii*; AZA 0.01% + PH: treated with 0.01% azadirachtin (in 1% NeemAzal T/S) and inoculated with *P. halstedii*; AZA 0.1% + PH: treated with 0.1% azadirachtin (in 10% NeemAzal T/S) and inoculated with *P. halstedii*. Bold values indicate significant ( $p \leq 0.05$ ) correlations. Based on Schober et al. [43]: 0.00 < 0.10: negligible correlation; 0.10 < 0.39: weak correlation; 0.40 < 0.69: moderate correlation; 0.70 < 0.89: strong correlation; 0.90 < 1.00: very strong correlation.

#### 4. Discussion

This research evaluated the effectiveness of neem-derived pesticides in reducing the symptoms and signs of downy mildew caused by two isolates of *P. halstedii* in susceptible sunflowers. Additionally, we examined the resistance induced by neem in the treated plants.

Our findings indicate that neem-based treatments significantly reduced disease progression, as reflected by lower disease rates and damping-off in treated plants compared to untreated controls. In a recent study, Doshi et al. [21] demonstrated that pre-treatment with neem leaf extract and azadirachtin, except at the lowest azadirachtin concentration (AZA 0.01%), prevented the development of disease symptoms in an aggressive *P. halstedii* isolate. Furthermore, they showed that these botanical pesticides, except AZA 0.01%, had significant antimicrobial activity against this isolate because they inhibited the release of zoospores. Using the same neem formulations and concentrations, our findings mostly align with those of Doshi et al. [21] on the disease-decreasing effect of these botanical pesticides, except that AZA 0.01% was as effective as the other treatments against two isolates of *P. halstedii* in the present study. However, only one *P. halstedii* isolate was used by Doshi et al. [21] without histopathological examinations. More recently, Berisha et al. [39] found that azadirachtin at a very low concentration (0.001%) failed to control downy mildew in sunflowers in a laboratory experiment, even when a lower concentration (35,000 instead of 50,000 sporangia/mL) of the pathogen inoculum was applied. Therefore, it seems that a concentration of 0.01% azadirachtin represents a limit of efficacy against sunflower downy mildew, which may be influenced by various factors (pathogen isolate, environmental conditions, etc.). Another possible reason could be that the treatment time was not long enough to observe an effect due to the very low concentration of azadirachtin in the treatment, such as AZA 0.001%. However, we recommend an extended treatment time for such low concentrations, i.e., more than two hours. In particular, in the NLE 10% treatment, plants inoculated with isolate 1 demonstrated significantly lower infection in the second evaluation than in the first evaluation. One reason for this is that the disease symptoms that emerged at the first evaluation did not propagate to the true leaves where the second evaluation was performed. This could be attributed to the systemic effect of neem, as also

stated by Doshi et al. [21]. We recommend testing plant samples to confirm the presence of different neem compounds in order to validate their systemic effects. However, none of the NLE 10%-treated and inoculated (isolate 1) plants died during the experiment (Table 1), which also impacted the disease rate. This effect of NLE 10% could be attributed to the different compounds, in addition to azadirachtin, present in the neem leaves [21]. Although different concentrations of such compounds were not identified and quantified in this study, we recommend testing the effects of different compounds individually and in combination against *P. halstedii* in sunflowers to better understand their roles.

Although its use in field crops was banned in the EU in 2021 due to fungicide resistance problems [44], mefenoxam was employed as a control in this study because of its comprehensive efficacy. In particular, no significant differences were observed between the effectiveness of various neem concentrations and mefenoxam in the present and previous studies [21]. This suggests that, at least at the investigated concentrations and formulations, neem-based pesticides can be as effective as synthetic active ingredients in controlling *P. halstedii* infections. In their study, Oros and Ujváry [45] and Oros [46] additionally demonstrated that the activity of some plant extracts against sunflower downy mildew was comparable to that of metalaxyl, the earlier iteration of mefenoxam. Paul and Sharma [14] also found that neem's effect was analogous to carbendazim against *Drechslera graminea* in barley. These findings hold promise for the future of plant pathology and agriculture, offering a sustainable and effective solution for integrated pest management.

One of the most severe symptoms of sunflower downy mildew is plant growth retardation, which culminates in dwarfed plants. The exact reasons for this phenomenon are still unclear, likely due to changes in nutrient uptake and hormonal manipulation caused by pathogens [36,47]. When examining plant height in our investigation, we found that the growth-promoting effects of neem were evident in the treated and inoculated plants. Specifically, the height of plants treated with various neem-derived pesticides and inoculated with different isolates was significantly greater than that of non-treated (inoculated) plants, matching the height of treated but non-inoculated plants. The only exception was in plants treated with 0.01% azadirachtin (AZA 0.01%) and inoculated with isolate 2, where the lower azadirachtin concentration failed to mitigate the dwarfing effect of *P. halstedii*. Similar results have been documented in other studies, where neem treatments were found to enhance plant vigor and growth, probably due to their ability to activate defense mechanisms that reduce pathogen-induced stress [12,15,16].

Histopathological analysis in our work provided further insights into the mechanisms of action of neem-derived pesticides. The presence of *P. halstedii* hyphae in the cortical tissue of sunflower hypocotyls was detected in small amounts with most treatments, except for plants treated with mefenoxam and AZA 0.1%, which exhibited significant hyphal growth in the cortical tissues when inoculated with isolate 1. This suggests that while neem treatments (and mefenoxam) helped reduce the spread of *P. halstedii*, they did not fully inhibit pathogen colonization, which is similar to the findings of Singh and Prithiviraj [19], Paul and Sharma [14], and Farag Hanaa et al. [16], where neem treatments were associated with partial but effective inhibition of growth of other fungal pathogens.

Interestingly, in our experiment, apart from mefenoxam-treated plants inoculated with isolate 1, necrosis (hypersensitive cell death) in infected tissues was similar between the control (non-treated) and neem-treated plants. This observation appears to diverge from the finding that disease rate and damping-off were significantly lower for all treated plants than for non-treated plants when assessing disease symptoms (Figure 2, Table 1). The most likely reason is that host-parasite histological analysis is only possible on live (surviving) plants, which were markedly limited among the non-treated plants. The few non-treated plants that survived were not necessarily extensively infected, consequently

enabling similar histological changes (necrosis) to be quantified across both treated and non-treated plants. Consequently, it should be emphasized that the results of symptomatic and histopathological examinations require integrated interpretation to obtain comprehensive insights into pathogenic interactions.

The relatively small amount of necrosis detected in our study for the treated plants contrasts with the results of Singh and Prithiviraj [19], who documented higher rates of hypersensitive cell death in pea leaves treated with neem and inoculated with *Erysiphe pisi*. Furthermore, our work observed a weak negative correlation between tissue hyphae and necrosis only in mefenoxam and AZA 0.1% treatments with isolate 1 and AZA 0.01% treatments with isolate 2. This observation contradicts that of Nisha et al. [48], who reported robust positive correlations between the presence of hyphae in the cortical parenchyma tissues and the appearance of necrosis in mefenoxam-treated sunflowers inoculated with *P. halstedii*. Constrained necrosis in treated plants potentially originates from the multifaceted immune mechanisms of plants, where effective resistance does not invariably necessitate cell death activation [28,30].

Our results align with those of Mouzeyar et al. [32] in observing post-penetration resistance; that is, pathogen entry occurs but is later blocked. Additionally, neem can mimic the genetic defenses detected in mildew-resistant sunflowers [33], although the exact mechanisms (e.g., cell wall changes and signal cascades) may differ. The histopathological features (e.g., cell necrosis) in neem-treated sunflowers in our study were less pronounced than those in genetically resistant plants to *P. halstedii*. This suggests that neem-induced resistance may rely more on moderate or early defense (e.g., hormonal or biochemical priming) rather than localized cell death.

The two aspects of plant hypersensitive reactions, resistance and cell death, can be physiologically, genetically, and temporally separated. K nstler et al. [29] analyze that early defenses result in extreme resistance without any symptoms, while a moderately early defense response leads to resistance with controlled and limited cell and tissue death. In our study, the limited development of necrosis in neem-treated (inoculated) plants suggests that neem pesticides can improve plant resistance without inducing higher levels of hypersensitive cell death (necrosis), forming a moderate early defense response against *P. halstedii* infection. Although some critical studies have identified elements involved in the resistance process induced by neem, such as increased levels of antioxidant enzymes [16] and elevated levels of phenylalanine ammonia-lyase [14,19], further cellular, histological, and biochemical studies are necessary to fully understand the mechanisms underlying neem-induced resistance. Additionally, it is essential to conduct studies that enhance our understanding of how fungal effectors and elicitor proteins manipulate host plant processes to promote infection or activate defense responses [49–51]. These findings indicate a complex and dynamic interaction between fungal effectors and host defenses, highlighting potential molecular targets for sustainable crop protection strategies.

## 5. Conclusions

Our study highlights the potential of neem-derived pesticides in controlling sunflower downy mildew (*P. halstedii*). The results demonstrated that neem-derived plant protection product treatments significantly reduced symptoms and improved plant growth compared to mefenoxam treatment, a historically prevalent synthetic fungicide. Additionally, histopathological results suggest that neem-derived formulations may enhance sunflower resistance without inducing excessive hypersensitive cell death, supporting the hypothesis of moderate early defense response.

Although our findings are promising, further research is needed to refine the rate and time of application of neem-based pesticides, particularly under field conditions where

environmental factors may influence efficacy. Future studies should focus on optimizing neem oil concentration and treatment duration and assessing the long-term impacts of neem treatment on plant health and crop yield. Neem-derived products could be crucial for sustainable and integrated disease management strategies in sunflower cultivation.

Further cellular, histological, and biochemical studies are necessary to clarify the mechanisms underlying neem-induced resistance, particularly those not involving hypersensitive cell death. Neem treatments did not fully inhibit pathogen colonization, indicating partial resistance rather than complete immunity. Although neem-treated plants exhibited better growth, the mechanisms underlying growth promotion (e.g., hormonal effects) have not been investigated.

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